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14. ABSTRACT
Rho GTPases are molecular switches that fluctuate between on and off states. When active, these proteins function to remodel the actin cytoskeleton by interacting with a number of downstream effector molecules. Recent studies have linked the activation of Rho GTPases with the acquisition of a metastatic phenotype in many types of cancers especially breast cancer. This proposal incorporates a rational approach to target these signaling proteins using small molecule inhibitors that would interfere with their ability to become activated by Rho family guanine nucleotide exchange factors (RhoGEFs). We have developed a high throughput screening strategy identify novel inhibitors of Rho activation are currently following up on several compounds which appear to selectively inhibit Rho activation. These compounds may form the basis of future drug development strategies for the treatment of metastatic breast cancer.

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Annual Report 2005

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Breast Cancer Research Program Predoctoral Fellow
Proposal title: Rational Design of Rho Protein Inhibitors
Award # DAMD17-03-1-0646

Introduction

Rho GTPases belong to the Ras superfamily of small (~21 kDa) monomeric guanine nucleotide-binding proteins. There are approximately 20 Rho subfamily members, the most characterized of which are RhoA, Rac1, and Cdc42. Like other guanine nucleotide binding proteins, Rho family members are molecular switches that fluctuate between active GTP-bound and inactive GDP-bound states. Rho GTPases are signaling molecules that can propagate signal transduction events initiated by extracellular stimuli and have been shown to promote an invasive and metastatic phenotype in a variety of cancer types via cytoskeletal rearrangement, as shown in Fig. (1). For example, activation of receptor tyrosine kinases, G-protein coupled receptors, or integrin receptors results in the activation of a Rho Guanine nucleotide Exchange Factor (RhoGEF), which facilitates the GTP-loading of specific Rho GTPases via the catalytic Dbl-homology (DH) domain [for reviews, see 1-5]. Once GTP-bound, Rho GTPases adopt an active conformation and are free to interact with downstream effector molecules, resulting in their activation. Rho GTPases are mediators of actin cytoskeletal remodeling and have been linked with the acquisition of a metastatic and invasive phenotype in several cancer types [7-19]. Recently, numerous reports have suggested that Rho GTPase activation is an integral step during the invasion and metastasis process of a wide variety of cancers including inflammatory breast cancer (IBC) [7-19]. Consequently, a recent issue of the journal *Breast Cancer Research and Treatment* was devoted exclusively to reports describing the link between Rho family GTPases and breast cancer progression and dissemination [7-13]. Additionally, a recent report has shown the Rho isoform, RhoC, is essential for metastasis in a mouse breast tumor model [22]. The authors show that RhoC-deficient mice develop normally and have proper migratory function of neutrophils, thymocytes, T-cells, and B-cells. However, when subjected to a mammary breast cancer tumor formation model, which typically gives rise to palpable tumors at ~50 days and disseminated cancer in the lungs at ~70 days, the RhoC-deficient mice have a significant reduction in the amount of metastasis in the lung. These studies, as well as numerous others, suggest that interfering directly with Rho GTPase function is an ideal strategy for controlling the dissemination of primary tumors to distant metastatic sites. However, due in a large part to the lack of appropriate screening assays, there is currently a lack of useful small molecule inhibitors of Rho

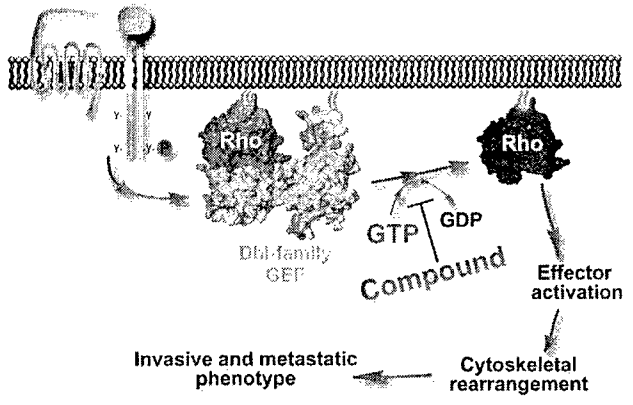

Fig. (1) Rho GTPases (Rho, Rac, Cdc42) are emerging candidates for targeted therapeutics in metastatic breast cancer; however, there are currently no compounds, which directly target the rate-limiting step of Rho activation. Activation of upstream signals, such as receptors, results in activation of Dbl-family guanine nucleotide exchange factors (GEFs) for Rho GTPases at the plasma membrane. GEFs catalyze the rate-limiting step of Rho activation by converting the inactive GDP-bound Rho and Rac into active GTP-bound Rho, which is then able to activate downstream effector molecules, resulting in cytoskeletal rearrangement, which contributes to an invasive and metastatic phenotype in many breast cancers. Our objective is to identify novel compounds, which disrupt the activation of Rho GTPases, thereby reducing the metastatic potential of cancer cells.

GTPase function, which could be developed into therapeutics for the treatment of metastatic breast cancer. For these reasons, our objective is to develop more efficient methods for high throughput screening and identification of novel inhibitors of Rho GTPase activation. This predoctoral fellowship encompasses a rational approach to target the oncogenic signaling properties of Rho GTPases in order to control aberrant signaling in breast cancer as well as other diseases. Our approach to discovering novel inhibitors of Rho protein signal transduction has encompassed both structure-based and high-throughput strategies and includes biochemical and cellular methods for subsequent characterization of lead compounds.

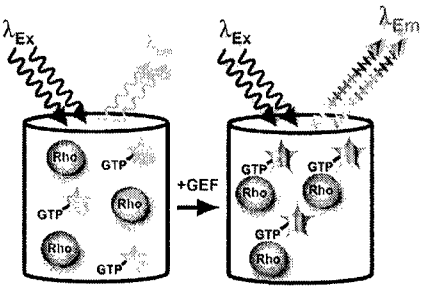

Fig. (2) The fluorescence-based guanine nucleotide exchange assay is used to monitor activation of Rho GTPases by RhoGEFs using purified components in real-time. Unbound fluorophore-conjugated guanine nucleotides ("GTP", left panel) are quenched in solution and emit a weak fluorescence signal upon excitation. However, when a GEF catalyzes guanine nucleotide exchange, the fluorophore-conjugated nucleotides bind to the Rho GTPase, and have an increased fluorescence emission upon excitation (right panel).

Results

***In vitro* guanine nucleotide exchange assay:** In order to conduct many of the proposed experiments outlined in the statement of work, we needed to first perfect a method for evaluating potential small molecule inhibitors of Rho GTPase activation. We have focused on a fluorescence-based guanine nucleotide exchange assay that used purified components and commercially available fluorescent probes. The guanine nucleotide exchange assay makes use of the spectroscopic differences between free and protein-bound fluorophore-conjugated guanine nucleotides as described in Fig. (**2**). Traditional methods for studying GTPase activation have relied on radiolabeled filter-binding assays, which are labor intensive, and lack precise sampling rates. However, the fluorescence-based exchange assay is more conducive for high content experiments that require precise determination of reaction rates. This is because real-time analysis, using a spectrophotometer, allows precise sampling rates required for determining the rate at which the exchange reaction occurs. While other groups routinely use this assay to characterize GTPases and GEFs, we have implemented this assay for the purposes of discovering novel inhibitors of Rho GTPase activation. This has entailed slight modifications to the original assay design, the details of which can be found in the methods section. Our assay validation studies have demonstrated that the assay is versatile enough to identify compounds with several different modes of action as described in Fig. (**3**). By using a functional assay, as opposed to a binding assay, we are not

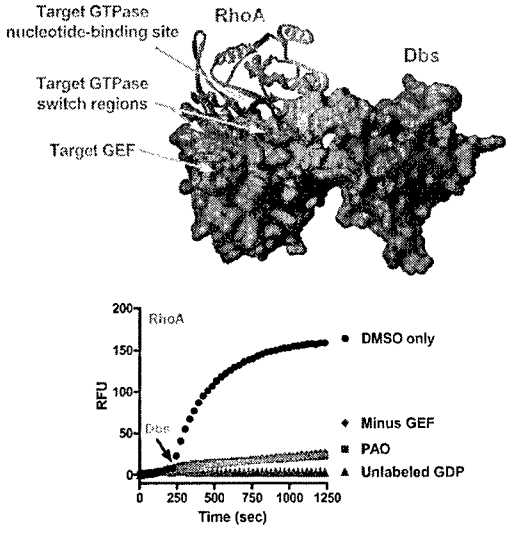

Fig. (3) The guanine nucleotide exchange assay can be used to identify compounds that inhibit Rho GTPase activation. Top panel shows the structure of nucleotide-depleted RhoA (green) bound to the catalytic region of the exchange factor Dbs (blue). Compounds that inhibit Rho activation may target the GTPase directly via the nucleotide-binding site (purple) or the switch regions (red); alternatively, compounds may directly target the GEF (blue). This assay is versatile because it can identify inhibitors that function via any of these mechanisms, as shown in bottom panel. Vehicle only control (DMSO) shows robust increase in fluorescence after addition of the GEF Dbs (arrow), while a covalent modifier of RhoA switch regions (PAO, phenylarsineoxide) inhibits RhoA activation. Additionally, a non-fluorescent guanine nucleotide (unlabeled GDP), which competes for the guanine nucleotide-binding site, as well as absence of GEF (minus GEF) also inhibit guanine nucleotide exchange.

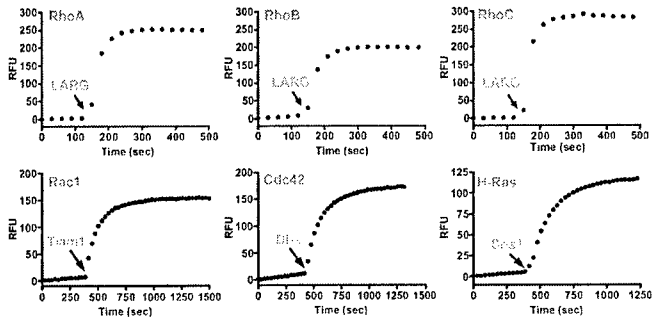

Fig. (4) We have applied the guanine nucleotide exchange assay to a large variety of Ras superfamily GTPases and their cognate GEFs. The GTPases RhoA, RhoB, RhoC, Rac1, Cdc42, and H-Ras were monitored for increase in fluorescence intensity upon addition of their appropriate GEF (LARG, Tiam1, Dbs, Sos1) at time indicated by arrow. We are using these purified protein reagents to determine the specificity of potential small molecule inhibitors as well as initiating new screening projects to identify additional compounds which disrupt signaling of other GTPase-GEF therapeutic targets.

limited by the mode of action any compound examined. Functional assays, such as the guanine nucleotide exchange assay, are therefore much more amendable to high throughput screening strategies. Additionally, we have purified a large number of Ras superfamily GTPases as well as their associated GEFs to near homogeneity for use in the guanine nucleotide exchange assay. This has entailed a high content approach to the cloning, expression, and purification of affinity-tagged protein constructs as described in the methods section. We are currently using these purified protein reagents to determine the selectivity of any identified small molecule inhibitors of Rho GTPase activation and have successfully used many in the guanine nucleotide exchange assay as shown in Fig. **(4)**. Furthermore, these additional protein reagents will form the basis of future screening programs aimed at identifying compound inhibitors for other GTPase/GEF therapeutic targets, such as Ras and Leukemia-associated RhoGEF (LARG).

***In silico* docking studies:** We have completed all of the proposed *in silico* docking experiments as outlined in the proposed statement of work and have completed most of the follow up experiments to determine which compounds have activity against Tiam1-mediated activation of Rac1. As detailed in the previous annual review, this work has included the characterization of the residues required for activity using site-directed mutagenesis and the subsequent *in silico* docking of solvent accessible sites surrounding key residues using a library of over 300,000 virtual compounds. We proposed to test ~100 of the top scoring compounds and have completed testing for only 41 of the highest scoring compounds predicted to inhibit Tiam1, the results of which are illustrated in Fig. **(5)**. Predicted inhibitors were first assayed for activity against Tiam1-Rac1 and many compounds interfered with the assay resulting in apparent increase in the rate of exchange, as indicated by a negative value for % inhibition, or showed no activity. Only four compounds had activity of greater than 30% inhibition at 100 μM. However, when subjected to a secondary screen, these compounds were found to be non-selective inhibitors as they also inhibited, to varying degrees, Sos1-mediated activation of H-

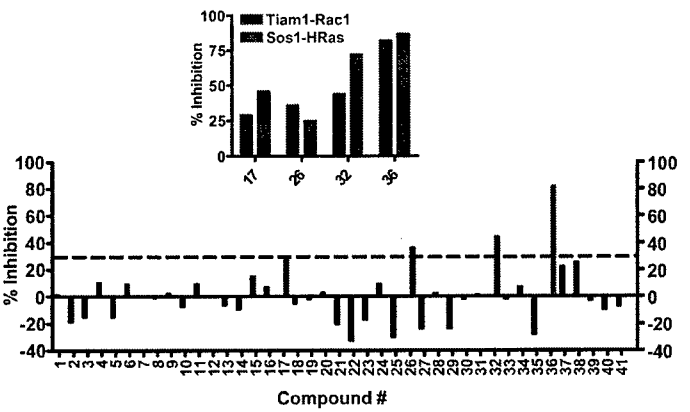

Fig. (5) Analysis of predicted inhibitors of Tiam1-mediated activation of Rac1. *In silico* docking was used to virtually screen a library of over 300,000 compounds for their ability to bind key residues on the Tiam1 protein surface implicated in activation of Rac1. These compounds were then obtained and tested in a functional guanine nucleotide exchange assay in order to determine their ability to inhibit Tiam1-mediated activation of Rac1. Bottom panel shows activity of 41 of the highest scoring predicted inhibitors obtained by docking. Four compounds (red bars) showed greater than 30% inhibition compared to DMSO only (dotted line) at 100 μM. However, these compounds were not selective as secondary screens using an unrelated GEF-GTPase pair, Sos1-HRas (upper insert).

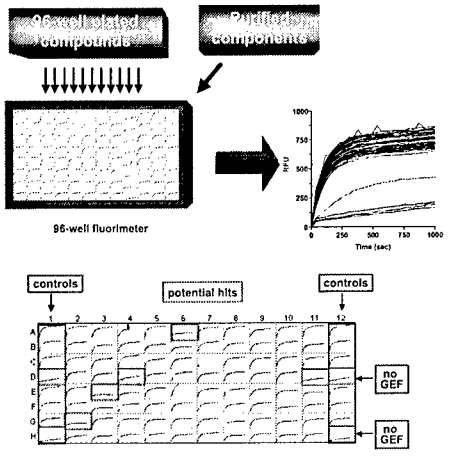

Fig. (6) A high throughput screening strategy to compliment existing structure-based approaches. Upper panel, purified components are added to a microtiter plate using a liquid handling robot, then assayed for guanine nucleotide exchange using a microplate-formatted fluorimeter. The results of each plate are graphed in order to determine the rate of exchange for each compound compared to DMSO controls. Compounds the inhibit exchange will have slower reaction rates (red curve) and will more closely resemble the control exchange curves for GTPase alone (blue curves). An example of data output from a recent screen is shown in bottom panel. Wells consisting of DMSO addition, or absence of GEF (blue boxes) are used as controls. Five potential inhibitors of guanine nucleotide exchange are shown in red boxes as an example of potential hits.

Ras as shown in Fig. (**5**), therefore these compounds will not be subjected to crystallographic analysis. Unfortunately, these docking studies have not yet produced a specific Tiam1 inhibitor as anticipated. Drug screening by means of small molecule docking is an ever-evolving technology that is in some ways superior to traditional methods for drug discovery, yet still retains many drawbacks [for a review, see 6]. A key disadvantage is the reliance on computational scoring algorithms for the characterization and ranking of potential inhibitors. In our case, it appears that the protein site used for docking purposes did not yield compounds with high enough affinities to compete with Rac1. Additionally, we may not have characterized enough of the predicted hits using the guanine nucleotide exchange assay. To address these concerns, we will screen additional predicted hits in the future and try docking novel protein sites at the binding interface of Tiam1. We may also try docking other RhoGEF structures, such as Dbs or LARG or conversely, the GTPase structure itself in order to generate a new list of potential inhibitors. While many resources were employed in order to identify novel compounds, we also have gained substantial information detailing residues required for Tiam1 activation of Rac1, as detailed in the last annual review, and have also gained a new skill set that can be applied to additional protein targets in the future. More importantly, we may use the techniques of virtual screening to optimize compound inhibitors identified using the complementary approach of high throughput screening.

High throughput screening-NCI Diversity Set: We have also incorporated additional strategies for the identification of small molecule inhibitors of Rho GTPase activation, namely high throughput screening of 96-well plated small molecule libraries as described in the original proposal. We have obtained and subsequently screened the National Cancer Institute (NCI) Diversity Set of ~2,000 compounds. This library is freely available to investigators and is designed to represent a wide variety of diverse compounds for screening purposes. We have completed screening for chemical modulators of Tiam1-mediated activation of Rac1 using a 96-well format of the guanine nucleotide exchange assay as outlined in Fig. (**6**). After the primary screening results, 32 compounds showed significant activity against Tiam1-mediated activation of Rac1. However, upon secondary analysis using non-related GEF-GTPase pairs, including Dbs-RhoA and Sos1-HRas, only one compound selectively modulated Tiam1 activity. Interestingly, this compound increases the kinetic rate of guanine nucleotide exchange and is therefore considered an activator, as opposed to an inhibitor as shown in Fig. (**7**). While our original purpose was to identify inhibitors of Rac1 signaling in order to intervene with metastatic signal transduction events, the discovery of a Tiam1 activator may prove useful as a biomolecular tool for probing Rac1-mediated signaling. As shown in Fig. (**7**), NSC#13778 (phenylstibinoacrylic acid) accelerates Tiam1 activation of Rac1 in a dose-dependent manner with an EC_{50} of ~5 μM, while unaffecting Dbs-RhoA, Dbs-Cdc42, and Sos1-HRas guanine nucleotide

exchange (data not shown). This activity has additionally been confirmed using non-fluorescent assays such as the traditional radiolabeled nucleotide filter-binding assay also shown in Fig. (7). We also have completed preliminary structure-activity relationship studies with over 15 structural analogs of NSC#13778, described at bottom of Fig. (7), in order to better understand which functional groups are most important for the mechanism of action. We are currently using X-ray crystallography to determine the molecular mode of action by isolating protein crystals of NSC#13778 bound to the nucleotide-depleted Rac1-Tiam1 complex.

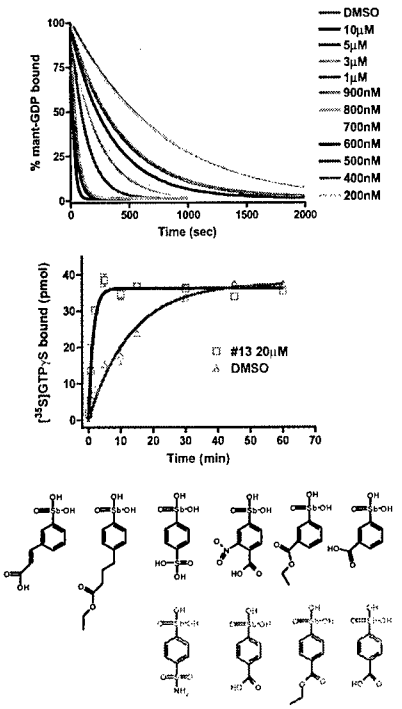

Fig. (7) High throughput screening of the NCI Diversity Set has yielded an activator of Tiam1. After screening ~2,000 diverse compounds, only one (NSC#13778) had selective activity as an activator of Tiam1 exchange on Rac1. Shown at top is a dose-response titration of NSC#13778 using a mant-GDP preloaded assay, where a decrease in fluorescence over time indicates Rac1 activation by Tiam1. As shown, NSC#13778 activates the exchange potential of Tiam1 in a dose-dependent manner with an EC_{50} of ~5 μM. This result has been confirmed in a number of secondary assays, including a radiolabeled guanine nucleotide filter-binding assay also pictured. Bottom panel of structures shows all structurally related active compounds in black, and structurally related inactive compounds in red. The highest affinity compound is the primary hit NSC#13778 at far left, with other compounds with less activity are also shown in black.

High throughput screening-LDDN collaboration: While the NCI Diversity is a useful library for initial drug screening purposes; it is of limited size and was mainly used as a proof of principle, in order to establish that our fluorescent nucleotide exchange assay can be used to identify novel small molecules that modulate Rho GTPase activation. We have therefore formed a collaborative agreement with an academic drug-screening lab, which gives our group access to over 60,000 small molecules in microtiter format. In light of recent reports implicating Rho in breast cancer metastasis [22], we have also decided to focus more intently on identifying Rho-specific inhibitors. For this reason, our current drug screening efforts have utilized Rho-specific GEFs, such as Dbs, instead of Tiam1 as originally proposed. Additionally, in order to effective screen such a large library, we needed to slightly modify the primary assay for ultra high throughput drug screening purposes, the details of which we hope to publish in the near future. Together with Dr. Ross Stein at the Laboratory for Drug Discovery in Neurodegeneration (LDDN), Harvard University, we converted the 96-well formatted fluorescent-based guanine nucleotide exchange assay, which was originally used to screen the NCI Diversity Set, into a simplified 2-point assay for 384-well plates in a semi-automated fashion. This has entailed slowing down the reaction kinetics in order to focus on the near linear portion of the exchange reaction and boost the overall signal as discussed in Fig. (8) and methods section. The linearized exchange reaction was then further simplified to a 2-point assay, vastly reducing the amount of manipulations, sampling rate, and post-screening analysis required to interpret results. An initial test set consisting of ten 384-well plates was then used to determine the statistical parameters typically employed to validate high throughput screening assays, as shown in Fig. (8) and described by Zhang et al. 1999. This test set illustrates that our method yields reproducible and statistically sound results, as the Z-factor is within the allowed range for an exceptional screening assay which is typically 0.6 - 1.0

[23]. We have completed screening ~30,000 compounds through this collaboration and are currently evaluating these results. As shown in Figure (**10**), most compounds are not active against Rho activation by Dbs, however 0.23% show significant activity of greater than 75% inhibition, as determined by equations illustrated in Fig. (**9**). This low hit rate is ideal for large screening projects because it allows every hit to be followed up using dose-response analysis and specificity studies, which are currently in progress. Due in large part to the success of our high throughput screening approach, we will soon be screening other therapeutic GTPase targets such as LARG (Leukemia Associated RhoGEF) using these libraries.

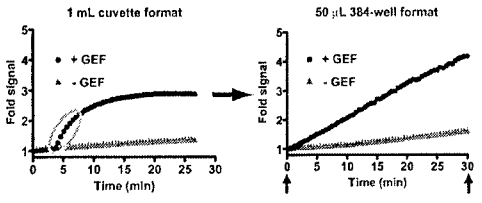

Fig. (8) We have converted our standard guanine nucleotide exchange assay into a simplified 2-point format for increased throughput and use in 384 well plates. Left panel shows the typical exchange occurring in a 1 mL total volume in a single quartz cuvette. This format was converted into a linear, low volume assay (right panel) by focusing on the near linear portion of the kinetic reaction (orange circle). Adjusting reaction components such as [Mg^{++}], [GEF], [GTPase], [fluorescent nucleotide], and pH resulted in slowing the reaction kinetics and boosting the overall signal. Conversion of the assay to a linear plot allows for further reduction to a simplified 2-poiint assay at time T=0 and T=30 min (arrows).

Training: As a predoctoral fellow in the Breast Cancer Research Program, I have received exceptional mentoring and formal training in a number of subject areas that will support my future as a cancer researcher. Foremost, my advisor John Sondek, Ph.D. has been instrumental in developing my skills in scientific inquiry by persistently challenging and encouraging my development as scientist. Aside from typical sources of instruction, which include formal group meetings and weekly seminars, my training has also included instruction in bioinformatics, computational techniques, three dimensional structure manipulation, virtual small molecule docking, protein purification techniques, high-throughput assay development, and X-ray crystallographic techniques for structure determination. This training regimen has consisted of formal training either individually from experts in the field or in a group setting in the form of course work and workshops. In addition to my advisor, thesis committee, and members of the department, I have also been closely mentored by several other outstanding researchers including the director of the UNC structural bioinformatics core facility (Brenda Temple, Ph.D.), members of the LDDN at Harvard University (Ross Stein, Ph.D. and Li-An Yeh, Ph.D.), the director of the X-ray core facility at UNC (Laurie Betts, Ph.D.), and the director of the protein core facility at UNC (Jason Snyder, Ph.D.). This training and mentorship has been invaluable to my development as a cancer researcher and has also significantly facilitated the implementation of these studies.

Methods

Protein purification: The coding sequences for all clones were introduced into a bacterial expression system as His-tagged protein constructs using standard PCR and molecular biology techniques. These clones consist of full-length sequences for the human GTPases RhoA, RhoB, RhoC, Rac1, Cdc42, and H-Ras as well as the catalytic fragments of the exchange factors Tiam1, Dbs, LARG, and Sos1. Proteins were expressed in E. coli and purified using standard Ni-NTA affinity purification methods followed by size-exclusion chromatography using an FPLC (fast performance liquid chromatography). All purified proteins were then subjected to SDS-PAGE analysis to determine correct molecular weight and protein purity. Only proteins that were >95% pure were used for biochemical and biophysical studies.

General guanine nucleotide exchange assays and testing of *in silico* predicted Tiam1 inhibitors: In order to monitor activity of GEFs, we performed a fluorescence-based guanine nucleotide exchange assay, as described in [20]. Proteins were constituted in 1X exchange buffer consisting of 10% glycerol (v/v), 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 10 mM $MgCl_2$. Conditions for

the cuvette-based fluorescence guanine nucleotide exchange assay consisted of 2 μM GTPase, 500nM BODIPY-conjugated GTP, and 200-400 nM GEF in 1X exchange buffer at total volume of 1,000 μL. Cuvettes were equilibrated at 20°C and stirred constantly. After ~500 sec of pre-incubation without GEF, the appropriate amount of GEF was manually pipetted into the reaction mixture initiated the reaction. Fluorescence was measured in real-time using a Perkin-Elmer LS-55 with excitation wavelength of 590 nm and emission wavelength of 620 nm. This protocol was used to determine the activity and selectivity of compounds predicted to inhibit Tiam1 based on *in silico* docking studies, which were described in the last annual report. Predicted inhibitors were purchased from commercially available sources and reconstituted in 100% DMSO solutions at a concentration of 10 mM. Any additional dilutions were also carried out in 100% DMSO to maintain solubility of compounds. Compounds were then added to exchange reactions containing either Tiam1-Rac1 or Sos1-HRas at a final concentration of 100 μM. Controls consisted of 1% DMSO additions, which did not alter exchange activity. Activity was determined by fitting each exchange reaction to an exponential growth curve using the graphing software GraphPad Prizm. The experimentally observed exchange rate (K_{obs}) for each tested compound was then compared to that of DMSO controls in order to obtain % inhibition values for each compound. All compounds from the *in silico* screen with greater than 30% activity against Tiam1 were analyzed extensively for dose-dependent activity and selectivity using additional exchange reaction substrates, including Sos1-HRas and Dbs-RhoA.

Fig. (9) A test set of ten 384-well plates was used to quantify and monitor the statistical significance of the 2-point screening assay. The Z-factor statistical parameter, commonly used for high throughput assays, and coefficient of variance (CV) was determined for each test plate using equations shown below.

	DMSO Control (N = 32/plate)		10 μM Compound (N = 352/plate)	
plate #	Z'-Factor	CV %	Z-Factor	CV %
1	0.57	9.04	0.65	6.60
2	0.63	7.19	0.59	7.64
3	0.65	7.03	0.53	9.89
4	0.65	6.93	0.60	7.91
5	0.75	4.99	0.59	9.26
6	0.67	6.24	0.59	8.00
7	0.63	7.65	0.61	7.94
8	0.66	7.17	0.64	7.63
9	0.73	5.60	0.67	7.29
10	0.74	5.18	0.63	8.24
mean:	0.67	6.70	0.61	8.04

Equations:

$$\text{Signal} = 30 \text{ min RFU} - 0 \text{ min RFU}$$

$$\%\ \text{Inhibition} = 100\left(1 - \frac{(\text{signal}_{\text{compound}} - \text{signal}_{\text{no GEF control}})}{(\text{signal}_{\text{DMSO}} - \text{signal}_{\text{no GEF control}})}\right)$$

$$CV\ \% = (100)\frac{SD_{signal}}{mean_{signal}}$$

$$Z = 1 - \frac{(3\ SD_{signal} + 3\ SD_{\text{no GEF control}})}{(mean_{signal} - mean_{\text{no GEF control}})}$$

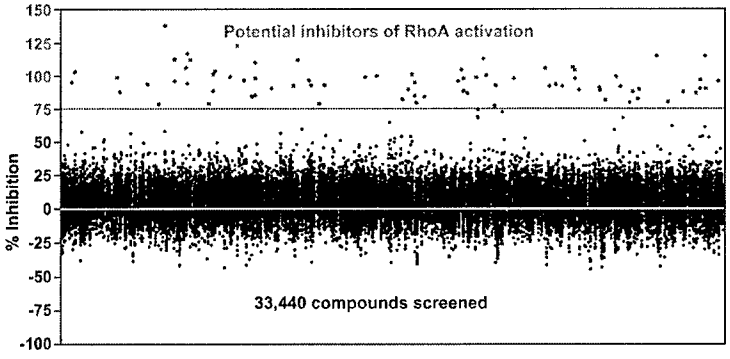

Fig. (10) A diverse set of compounds was screened in a high throughput format for inhibition of Dbs-mediated activation of RhoA using a semi-automated high throughput screening strategy in 384-well plates. Shown is a scatter plot of % inhibition for 33,440 compounds tested, which yielded a hit rate of 0.23% as defined as greater than 75% inhibition (red line). Also illustrated in red are hits that are currently being evaluated as potential inhibitors of RhoA activation.

High throughput screening of the NCI Diversity Set and analysis of NSC#13778: The publicly available Diversity Set was obtained from the NCI in a 96-well format and reconstituted in 100% DMSO at a final concentration of 10 mM. This library was used to screen for small molecule modulators (either activators or inhibitors) of Tiam1-mediated activation of Rac1 using reaction conditions described above. The screen was carried out in a microtiter, 96-well format using a Biomek FX liquid handling robot to set up the reactions and a Molecular Devices microtiter fluorimeter to monitor the reaction kinetics. All compounds tested were compared to DMSO only controls and the % inhibition was calculated by fitting each reaction to an exponential growth function using GraphPad Prizm data analysis software as described above. Secondary screening of potential hits consisted of screening compounds against an unrelated GEF-GTPase pair, such as Dbs-RhoA and Sos1-HRas in order to determine selectivity. A dose-response curve was then generated for NSC#13778 and was used to determine the EC_{50} value of ~5 μM.

High throughput screening collaborative effort with the LDDN and statistical validation of the 2-point assay: The 96-well formatted guanine nucleotide exchange assay used to screen the NCI Diversity Set was modified to suit the demands of a 384-well formatted screen of ~30,000 compounds by adjusting reaction conditions and using more sensitive instrumentation. The final reaction condition for screening compound at 10 μM consisted of 20 mM Tris pH 7.0, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 50 μg/mL BSA, 200 nM Dbs (catalytic DH/PH fragment), 1.5 μM RhoA, and 640 nM Bodipy-GTP (Molecular Probes). A Biomek FX liquid handling robot was used to add reagents to a top reading, black 384-well plate (Fluotrac 200). Fluorescence intensity was measured using an Analyst HT microplate fluorimeter (Molecular Devices) with an excitation wavelength of 580 nm, an emission wavelength of 630, nm and a bandpass filter at 600 nm. The signal was determined for each well by taking a zero time point immediately after initiating the reaction and at 30 minutes. Statistical parameters were then calculated for each compound using equations shown in Fig. (**9**) and described by Zhang et al. 1999 [23]. All hits that inhibit exchange greater than 75% are currently being followed up as potential inhibitors of RhoA activation.

Key Research Accomplishments

Virtual drug discovery

- Computational analysis of solvent accessible sites at the GTPase/RhoGEF interface of Rac1/Tiam1 and RhoA/Dbs
- Mutational analysis of Tiam1 residues required for binding and activating Rac1
- Reformatting of the Ryan Scientific small molecule library (>300,000 compounds) for virtual screening purposes; conversion from sdf format to mol2 3-D format
- Virtual screening of Tiam1 binding surface utilizing the algorithms DOCK and FlexX
- Analysis of virtual compound hits, and testing compounds for activity using a functional guanine nucleotide exchange assay and selectivity screens using unrelated GEF-GTPases

High throughput assay development and small molecule library screening

- Purification of large quantities of proteins (100-300 mg each) for use in high throughput screening and secondary studies, including Dbs, Tiam1, Sos1, RhoA, RhoB, RhoC, Cdc42, Rac1, and H-Ras
- Screening the NCI Diversity Set of ~2,000 compounds using a 96-well formatted fluorescence-based guanine nucleotide exchange assay
- Secondary characterization of NSC#13778, including selectivity and dose-response studies; structural studies of NSC#13778 are currently in progress
- Conversion of a kinetic fluorescence-based guanine nucleotide exchange assay to high throughput, 2-point assay in a semi-automated 384-well format for drug screening purposes
- Test screening ten 384-well plates for determination of statistical parameters and assuring assay validation
- Screening ~30,000 compounds for activity as chemical modulators of Dbs-mediated activation of RhoA through a collaboration with Dr. Ross Stein, Laboratory for Drug Discovery in Neurodegeneration, Harvard University; identified hits as a result of this screen are currently being evaluated

Reportable Outcomes

N/A

Conclusions

In conclusion, recent studies have further linked Rho GTPase activation to cancer progression and acquisition of a metastatic phenotype in many types of cancers including breast cancers [7-19].

We have completed most *in silico* docking studies; yet have not found any selective inhibitors using this method. For this reason, we have begun to focus more intently on a high throughput screening approach to discovering novel chemical modulators of Rho activation. This proposal incorporates a rational approach to target these signaling proteins using small molecule inhibitors that would interfere with their ability to become activated by RhoGEFs. To this end, we have initiated both a virtual drug screening strategy and a high throughput approach with the aim of identifying small molecule inhibitors that may be characterized using biochemical, as well as cellular methods.

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Appendices
N/A